LEUKEMIC CELLS SENSITIVE OR RESISTANT TO β-INTERFERON HAVE IDENTICAL GANGLIOSIDE PATTERNS

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1. Introduction

A β-interferon-resistant subline of mouse leukemia L-1210 cells (L-1210R) has been isolated and cloned after prolonged culture of L-1210 cells in the presence of this interferon [1]. Its resistance to antiviral and antigrowth effects of mouse β-interferon is caused by the absence of functional receptors [2,3]. The initial step in β-interferon action may involve binding to cell membrane gangliosides of target cells, since this interferon interacts with the carbohydrate portion of these glycolipids [4–6]. To investigate whether the absence of functional receptors on L-1210R cells correlates with a lack of specific gangliosides, we have compared the ganglioside pattern of parent L-1210 cells with that of its receptor-negative mutant, L-1210R. Our data show identical gangliosides in both types of cells and reveal the same proportions of *N*-acetylneuraminyl- and *N*-glycolylneuraminyl-G_{M3} in their cell extracts. Thus interferon resistance and lack of high affinity binding in L-1210R cells cannot be attributed to an altered ganglioside pattern in this subline.

Abbreviations: *N*-acetylneuraminyl-G_{M3}, 3-*O*-α-*N*-acetylneuraminyl-4-*O*-β-galactosyl-*O*-glucosyl-ceramide; *N*-glycolylneuraminyl-G_{M3}, 3-*O*-α-*N*-glycolylneuraminyl-4-*O*-β-galactosyl-*O*-glucosyl-ceramide; G_{M2}, 4-*O*-β-*N*-acetylgalactosaminyl (3-*O*-α-*N*-acetylneuraminyl)-4-*O*-β-galactosyl-*O*-glucosyl-ceramide; G_{D3}, 8-*O*-α-*N*-acetylneuraminyl-3-*O*-α-*N*-acetylneuraminyl-4-*O*-β-galactosyl-*O*-glucosyl-ceramide; G_{M1}, 3-*O*-β-galactosyl-4-*O*-β-*N*-acetylgalactosaminyl (3-*O*-α-*N*-acetylneuraminyl-4-*O*-β-galactosyl-*O*-glucosyl-ceramide; G_{D1a}, 3-*O*-α-*N*-acetylneuraminyl-3-*O*-β-galactosyl-4-*O*-β-*N*-acetylgalactosaminyl (3-*O*-α-*N*-acetylneuraminyl)-4-*O*-β-galactosyl-*O*-glucosyl-ceramide; G_{D1b}, 3-*O*-β-galactosyl-4-*O*-β-*N*-acetylgalactosaminyl (8-*O*-α-*N*-acetylneuramide-3-*O*-α-*N*-acetylneuraminyl)-4-*O*-β-galactosyl-*O*-glucosyl-ceramide; G_{T1b}, 3-*O*-α-*N*-acetylneuraminyl-3-*O*-β-galactosyl-4-*O*-β-*N*-acetylgalactosaminyl (8-*O*-α-*N*-acetylneuraminyl-3-*O*-α-*N*-acetylneuraminyl)-4-*O*-β-galactosyl-*O*-glucosyl-ceramide; Mu-IFN-β, murine β-interferon; TLC, thin-layer chromatography

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2. Materials and methods

2.1. *Cells*

L-1210 parent cells (L-1210S) and the Mu-IFN-β-resistant subline (L-1210R) were kindly provided by Ion Gresser. They were grown in RPMI 1640 medium (GIBCO) supplemented with 10% fetal bovine serum under static conditions at 37°C to a final density of 2–3 × 10^6 cells/ml. Just prior to harvesting large batches of cells, aliquots were removed and suspended to 10^5 cells/ml in RPMI 1640 medium plus 5% fetal bovine serum with or without 1000 NIH reference units/ml of Mu-IFN-β to assure unchanged sensitivity of L-1210S and resistance of L-1210R cells to its growth-inhibitory effect at the time of harvest. Cell counts after further 3 days of growth revealed 50% less L-1210S cells in the interferon-treated samples as compared to control counts, and no difference between interferon-treated and control L-1210R cells.

2.2. *Reagents*

Mouse fibroblast interferon was supplied by Kurt Pauker (2 × 10^7 NIH reference units/mg). Individual gangliosides were from Seromed (Munich). *N*-Glycolylneuraminyl-lactosyl-ceramide was kindly provided by Subhash Basu, (*N*-acetyl-neuraminyl)$_2$-lactosyl-ceramide (G_{D3}) was isolated from bovine brain [7]. *N*-Acetylneuraminic acid, *N*-glycolylneuraminic acid, lactosyl-ceramide and *Clostridium perfringens* neuraminidase were from Sigma.

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2.3. *Extraction of cells*

Cells (~10^9/batch) were isolated by centrifugation and washed repeatedly with cold PBS. The packed cells were then extracted with chloroform–methanol (2:1, 40 ml solvent/3 ml cell pellet) using a Brinkman Polytron for 10 min to homogenize the cells in the solvent. After centrifugation the same process was repeated twice followed by 3 more identical extractions using chloroform–methanol (1:2). Supernatants of all extractions were combined and concentrated on a rotary evaporator to a small volume, then taken to dryness in small conical tubes by a stream of nitrogen. Samples were stored at 0°C until further analysed.

2.4. *Neuraminidase treatment*

Commercial *Clostridium perfringens* neuraminidase was desalted on a small column of Sephadex G-50. Individual ganglioside samples (10–50 nmol) were digested with 1.3 units of neuraminidase in 0.1 ml water for 1 h at 37°C and lyophilized prior to thin-layer chromatography. Under the conditions used *C. perfringens* neuraminidase quantitatively removes sialic acid from authentic *N*-acetylneuraminyl-G_{M3}, *N*-glycolylneuraminyl-G_{M3} and G_{D3}, but does not hydrolyse G_{M2}- or G_{M1}-bound sialic acid.

2.5. *Other analytical procedures*

Thin-layer chromatography was done with poly(ethylene terephthalate)-backed silica gel sheets (Eastman Kodak Co.). The solvents used were chloroform–methanol–2.5 N NH_4OH (60:40:9, by vol., solvent 1) and chloroform–methanol–0.25% KCl (60:40:8, by vol., solvent 2). Sialic acid-positive spots were identified by resorcinol spray [8]. Ratios of individual gangliosides were determined after scraping corresponding resorcinol-positive areas, extracting with 0.5 ml butanol–butyl acetate (85:15, v/v) and determining the absorbance of the cleared extracts at

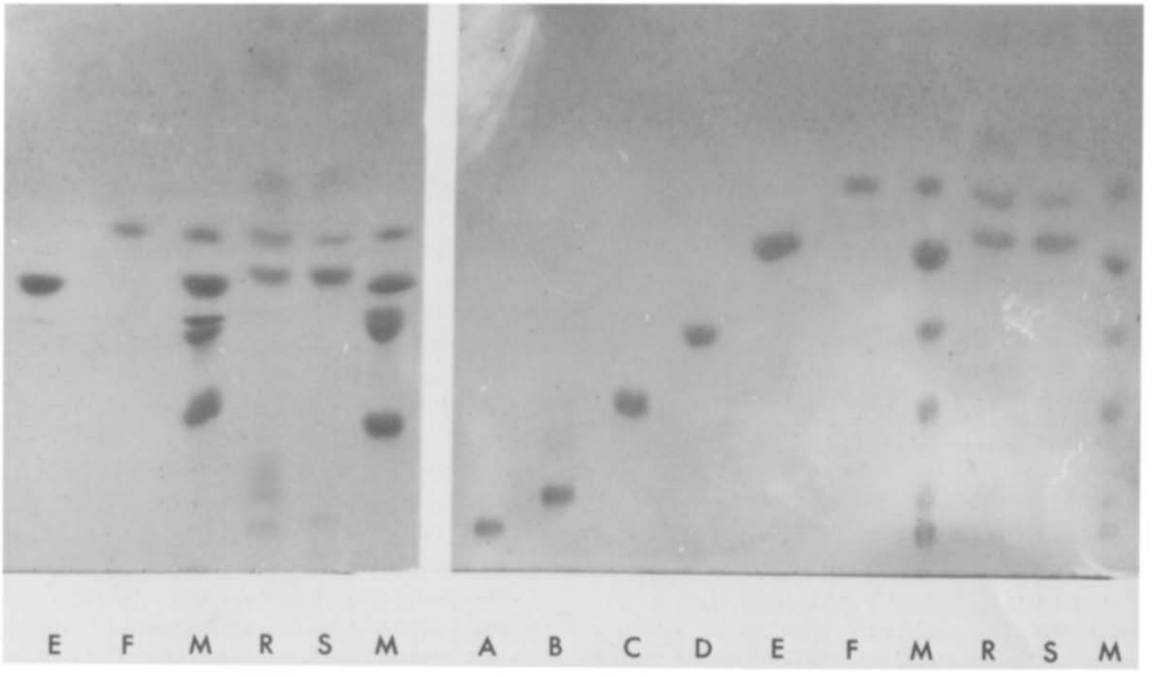

Fig.1. Separation of gangliosides from L-1210S (S) and L-1210R (R) cells by TLC in solvents 1 (left) and 2 (right). The standards are: (A) G_{T1b}; (B) G_{D1b}; (C) G_{D1a}; (D) G_{M1}; (E) G_{M2}; (F) *N*-acetylneuraminyl-G_{M3}; (M) mixture of (A–F). Di-sialoganglioside G_{D3} migrates slightly ahead of G_{M1} and slower than G_{M2} in solvent 2 (not shown). The only two blue-staining spots characteristic for sialic acid-containing glycolipids in R and S where those migrating like authentic *N*-acetylneuraminyl-G_{M3} and slightly ahead of G_{M2}. Faster-moving spots stained brownish-yellow and represent non-ganglioside lipid contaminants. Spots slower than G_{D1b} in solvent 1 represent traces of free sialic acids that arose during storage of the glycolipid extracts due to some degradation of the gangliosides and were absent on chromatograms of freshly prepared extracts (see solvent 2, right).

580 nm. Lactosyl-ceramide was localized by the α-naphthol spray [8]. Sialic acid was determined with resorcinol [9]. The same molar amounts of *N*-acetyl- and *N*-glycolyl-derivatives yield identical *A*-values at 580 nm [10]. Cell counts were done using a hemocytometer.

3. Results

Determination of total sialic acid in chloroform–methanol extracts yielded 0.6 g/10^6 L-1210S cells and 0.8 μg/10^6 L-1210R cells. This difference, most likely due to the error involved in individual cell counts, does not reveal a deficiency of lipid-bound sialic acid in L-1210R cells as compared to L-1210S cells.

There were only 2 detectable sialic acid-containing glycolipids in the extracts of either L-1210S or L-1210R cells (fig.1). The faster moving components from each cell type had identical mobilities in the neutral and the alkaline solvents used. The same was found for the slower moving components. The faster moving spots migrated identically to *N*-acetylneuraminyl-G_{M3}, and the slower moving spots slightly ahead of G_{M2} and identically to *N*-glycolylneuraminyl-G_{M3}, as shown below.

Extraction of individual resorcinol-stained spots with butanol–butyl acetate followed by spectrophotometric determination revealed 20 ± 3% of total resorcinol colour in the upper spots and 80 ± 3% in the lower spots for both L-1210S and L-1210R cell extracts.

The resorcinol-positive components of L-1210S and L-1210R cell extracts were separately isolated using preparative TLC. Extracts from 5 × 10^8 cells were applied in narrow lines on the TLC sheets and separated in solvent 2. Guide strips were cut off on each side of the sheets and developed with resorcinol spray. Zones on the non-developed sheets corresponding to resorcinol-positive areas were scraped and extracted with 1 ml chloroform–methanol (2:1) 3 times. Combined supernatants were brought to dryness and aliquots subjected to TLC. Complete separation of the glycolipids was achieved for solvent 1 (fig.2): the faster spots migrated like *N*-acetylneuraminyl-G_{M3}, and the slower spots identically to *N*-glycolylneuraminyl-G_{M3}. The same was observed in solvent 2 (not shown). Treatment with neuraminidase released *N*-acetylneuraminic acid from the faster moving glycolipids, and *N*-glycolylneuraminic acid from the slower-moving glycolipids, as indicated after TLC in solvents 1 (fig.3) and 2 (not shown). The α-naphthol spray revealed spots corresponding in mobility to authentic lactosyl-ceramide in all 4 neuraminidase-treated samples (solvents 1 and 2), which were absent in non-treated controls. Thus all 4 gangliosides have sialic acids as non-reducing terminals, the faster-moving ones contain *N*-acetylneuraminic acid, and the slower-moving spots *N*-glycolylneuraminic acid. Since neuraminidase treatment released lactosyl-ceramide from all 4 gangliosides, and since all 4 were chromatographically distinguishable from G_{D3}, it follows that the faster-moving spots are *N*-acetylneuraminyl-lactosyl-ceramide, and the slower-moving spots *N*-glycolylneuraminyl-lactosyl-ceramide.

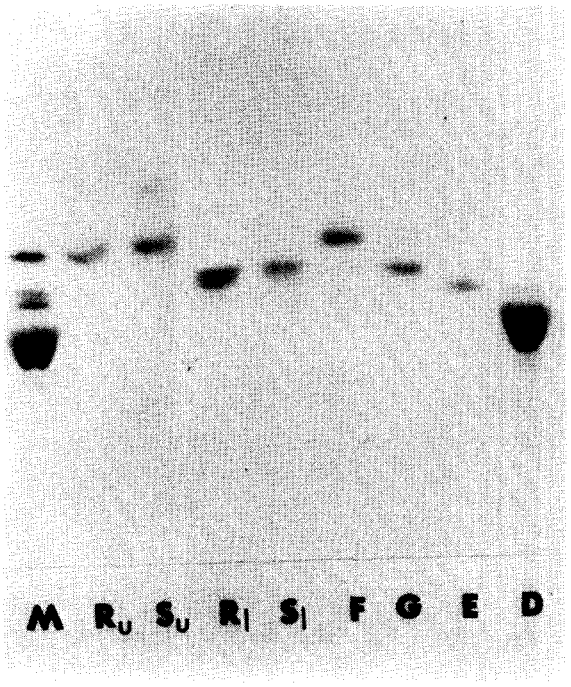

Fig.2. TLC of individually separated gangliosides from L-1210S and L-1210R cells in solvent 1. R_u and S_u are upper spots, and R_l and S_l lower spots from the respective cell extracts that were isolated after preparative TLC of each extract. The standards are: (D) G_{M1}; (E) G_{M2}; (F) *N*-acetylneuraminyl-G_{M3}; (G) *N*-glycolylneuraminyl-G_{M3}; (M) mixture of (D–G). Faint spots in R_u and S_u ahead of *N*-acetylneuraminyl-G_{M3} are brownish yellow-staining non-ganglioside contaminants.

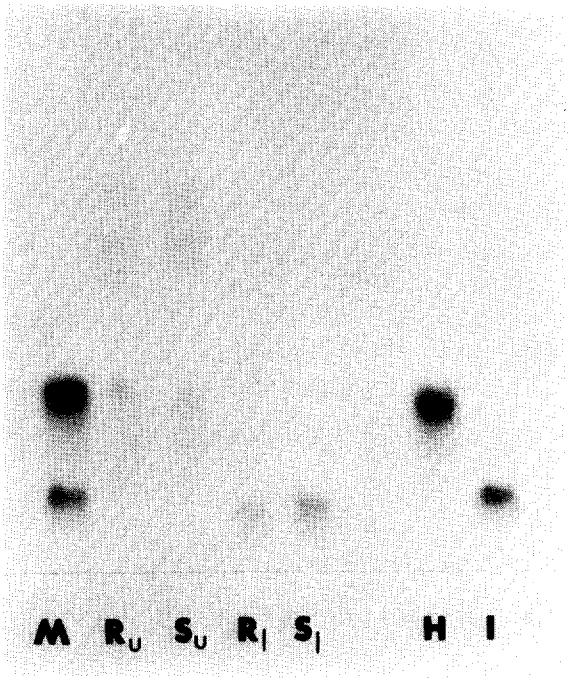

Fig.3. Separation of sialic acids released from individual gangliosides after treatment with neuraminidase: R_u and S_u, from the respective upper spots, and R_1 and S_1 from the respective lower spots of L-1210R (R) and L-1210S (S) cell extracts. The standards are: (H) *N*-acetylneuraminic acid; (I) *N*-glycolylneuraminic acid. Spots migrating ahead of *N*-acetylneuraminic acid in R_u and S_u are brownish yellow-staining contaminants.

4. Discussion

The ganglioside patterns of L-1210S cells and of the β-interferon-resistant subline L-1210R are very similar, qualitatively as well as quantitatively. The absence of higher gangliosides is comparable to other B-lymphocytic mouse tumor lines, which likewise lack significant amounts of gangliosides more complex than G_{M3} [11]. When exogenously added to target cells together with interferon, *N*-acetylneuraminyl-G_{M3} [6] and *N*-glycolylneuraminyl-G_{M3} (unpublished) are inhibitory to the antiviral action of Mu-IFN-β and thus can bind to it. Since carbohydrate residues of cell membrane gangliosides are exposed on the outside of the cell, binding of β-interferon to these carbohydrate residues could conceivably be the initial event that preceeds its biological action. However, if interaction of β-interferon with either or both of the gangliosides present in L-1210S cells was responsible for binding to its receptor, the same high affinity binding should also occur with L-1210R cells, as both cells show very similar ganglioside patterns.

The affinity of β-interferon for cell membrane gangliosides may not be sufficient to allow for high affinity binding to target cells and their carbohydrate chains may not participate in productive interaction with the interferon molecule. The dissociation constant for the receptor Mu-IFN-β complex on L-1210S cells is in the picomolar range (10^{-11} M in [2]). However, 10–100 μM ganglioside have to be employed to inhibit antiviral and antigrowth effects [6]. The fact that binding of β-interferon to L-1210S cells is trypsin-sensitive suggests that an important part of the receptor is protein or glycoprotein in nature [3]. Thus, the observed binding of β-interferon to isolated gangliosides might be the result of its affinity for carbohydrate side chains on sialic acid-containing glycoprotein receptors which are structurally similar to those found on gangliosides.

Acknowledgements

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